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TITLE: Modulators of Response to Tumor Necrosis-Related Apoptosis-Inducing Ligand (TRAIL) Therapy in Ovarian Cancer

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INTRODUCTION:

Ovarian cancer is the leading cause of death from gynecologic cancers in the developed world. Most ovarian cancers are diagnosed late and current treatment results only in a 20% 5-year survival in advanced disease. More effective therapies are urgently needed. One of the most promising therapies in development for ovarian cancer is the use of either the Tumor Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) or agonistic antibodies that activate the receptors for TRAIL. Both these strategies are designed to induce apoptosis in ovarian cancer cells. TRAIL therapies are particularly exciting because TRAIL reverses chemoresistance to standard chemotherapy as well as having a direct growth inhibitory effect on ovarian cancer cells, while sparing normal ovarian cells. However, the characteristics of ovarian tumor cells that determine whether TRAIL pathway agonists will be effective are poorly understood. For this reason, we currently do not have a rational basis for selecting patients who will benefit most from drugs that target this pathway or for improving the clinical response in those patients whose tumors are refractory to TRAIL pathway activators.

We have identified a homeobox gene, Six1, which is over-expressed in ovarian cancers as compared to normal ovarian surface epithelium. Expression of Six1 is correlated with poor clinical prognosis and confers resistance to TRAIL, possibly via upregulation of a decoy receptor. If this is the case, tumor cells would be expected to be resistant to TRAIL, but not to TRAIL agonistic antibodies. **We hypothesize that Six1 expression in ovarian cell lines and primary tumor cells results in resistance to TRAIL-induced apoptosis through activation of the DcR1 decoy receptor**.

Our specific aim is to (1) To confirm DcR1 as a downstream target of Six1 in ovarian cancer cells, (2) To determine if DcR1 expression is the mechanism by which Six1 expression regulates the response of ovarian cancer cells to TRAIL pathway agonists, and (3) To determine if Six1 expression regulates the response of cell lines derived from *primary* ovarian cancers to TRAIL pathway agonists.

We will test our hypothesis by verifying DcR1 as a downstream target of Six1, confirming loss of TRAIL sensitivity (versus no loss of sensitivity to TRAIL agonistic antibody or control) in a Six1 over-expressing ovarian cancer model, and expanding the analysis of TRAIL sensitivity to Six1 over-expressing primary ovarian cancer cell lines in culture and *in-vivo*. We expect that DcR1 (RNA and protein) will be increased with Six1 over-expression and decreased with Six1 siRNA knockdown, and that downregulation of DcR1 in Six1 over-expressing cell lines will inhibit the ability of Six1 to confer TRAIL resistance. We will provide evidence that Six1 directly regulates DcR1 expression by performing Gel shift, ChIP and promoter activation assays. Further, we expect to see loss of TRAIL sensitivity (as compared to control) in Six1 over-expressing transfected and primary cancer cell lines.

If our hypothesis is correct, it will have a profound implication for current Phase I studies of TRAIL and its agonistic antibodies in cancers (ovarian and others). Thus, Six1 over-expressing tumors are predicted to be resistant to TRAIL. With this knowledge, it may be possible to predict which cancers are TRAIL insensitive by virtue of their levels of Six1 expression, providing a way to select patients for TRAIL clinical trials that are more likely to benefit from this therapy. In addition, these studies should also provide a firm basis to develop strategies to reverse resistance to the TRAIL pathway, leading to the development of potential combination treatments that will improve the clinical response in patients with unfavorable prognoses.

BODY:

The following section is organized according to the proposed statement of work for the initial first year of the grant and accomplishments towards completing the task.

Statement of Work: Tasks for Year 1

Task 1. **Verify DcR1 as a target of Six1 (1-9 Months)**

a. Collect and propagate specimens and cell lines to complete Six1 RNA and DcR1 RNA and protein analysis.
b. Perform CaOV3-Six1 and SKOV3 siRNA experiments

Task 1. **Work Completed:**

Additional stable-expressing CaOV3-CAT clones CaOV3-Six1 clones were generated early in this task for a more-robust analysis. Analysis of CaOV3-Six1 over-expressing cell lines and cell lines from ovarian cancer patients did not confirm DcR1 as a downstream target (see Fig1-4 below). Furthermore, the SKOV3 cell line, which over-expresses Six1, did not over-express DcR1, making siRNA experiments not possible. However, another decoy receptor, DcR2 was up-regulated and is currently the focus of further studies.

Fig 1. 3 CaOV3 CAT clones are compared to 4 CaOV3-Six1 over-expressing clones. Six1 and DcR1 expression is evaluated by quantitative real-time PCR

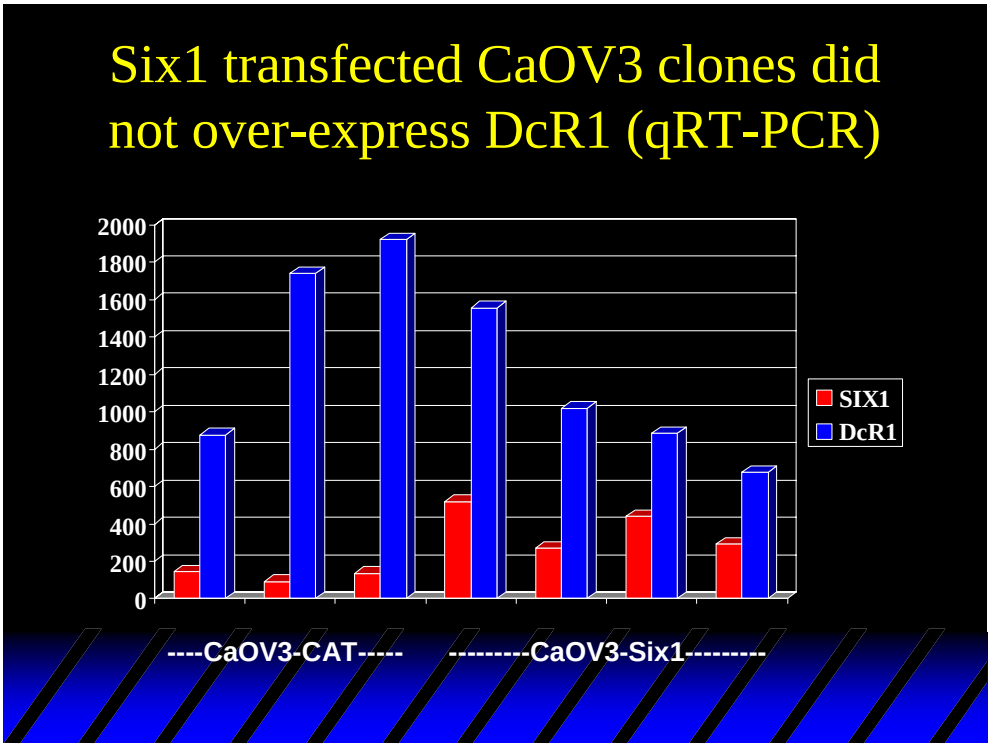


Fig 2. 2 newly generated CAOV3-CAT clones are compared to 2 newly generated CaOV3-Six1 clones and the Six1 over-expressing cell Line SKOV3. TRAIL receptor analysis is performed by flow cytometry. While DcR1 over-expression is not consistent in CaOV3-Six1 over-expressing cells, DcR2 is overexpressed, making it a better possible downstream target. The SKOV3 cell line also over-expresses DcR2

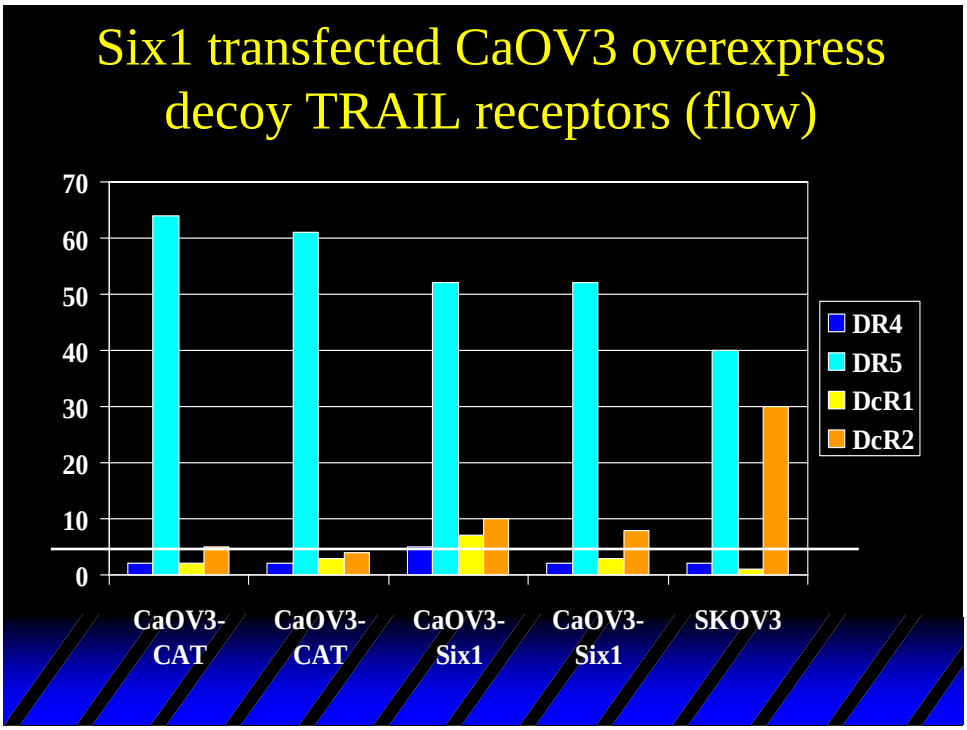


Figure 3. In support of Figure 2, analysis of DcR2 protein by western blot also demonstrates that DcR2 is over-expressed in CaOV3-Six1 clones

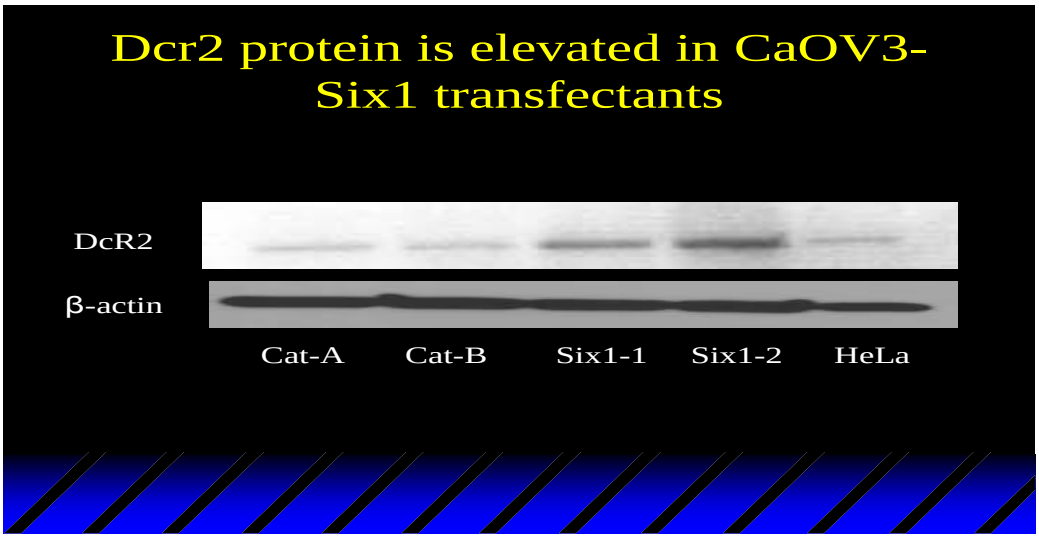
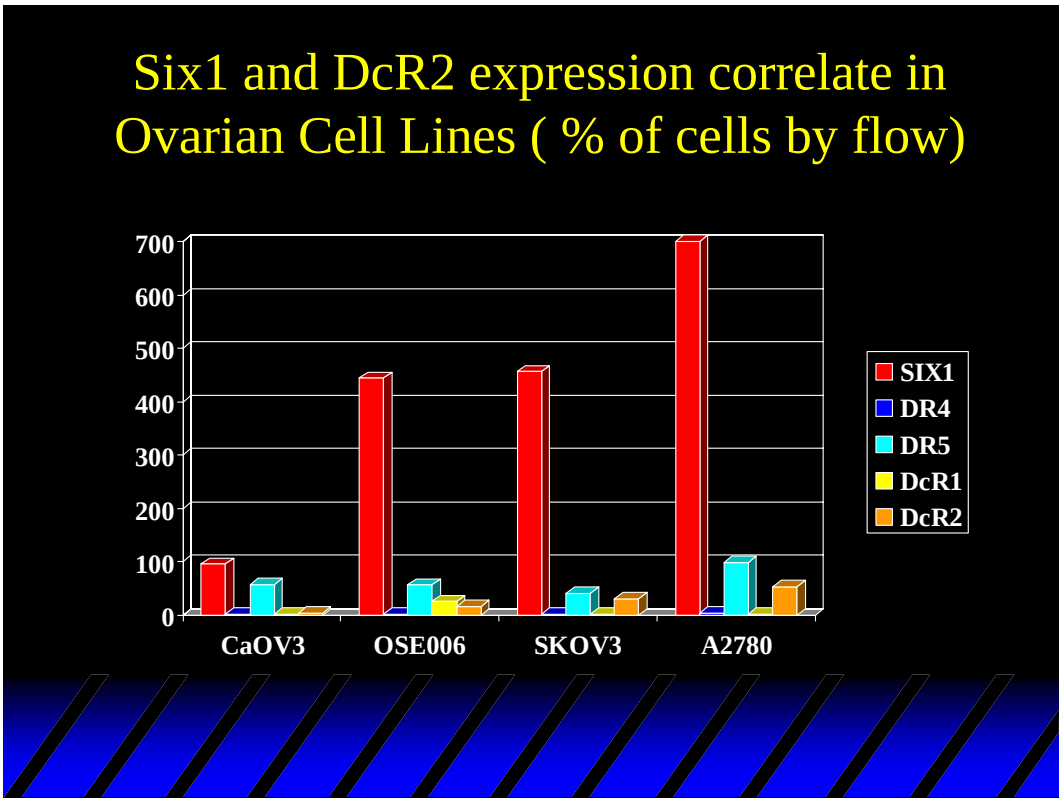


Figure 4. Analysis of additional commercially available cell lines shows a correlation between Six1 over-expression and DcR2 overexpression (by flow cytometry).



Task 2. **Determine whether DcR1 is a direct or indirect target of Six1 (Months 6-12)**

a. Gel Shift
b. Chromatin I.P. experiments
c. Promoter activation studies

Task 2. **Work performed**

The experiments planed for task 2 were halted after the results of task 1, We are currently preparing reagents and systems to perform the same experiments using DcR2 as a target (instead of DcR1).

Task 3. **Evaluate TRAIL panel sensitivity in Six1 over-expressing and knock-down cells (Months 1-18)**

a. Generate inducible models of Six1 expression
b. Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using existing CaOV3 **Six1**-over-expression model and SKOV3 **Six1** knock-down model, save cell pellets and extract RNA and protein.

Task 3. **Work Performed:**

Generation of inducible models for Six1 over-expression and knock-down are on-going.

Task 4. **Evaluate TRAIL panel sensitivity in primary ovarian cancers cell lines and correlate with Six1 and DcR1 expression (Months 6-18)**

a. Perform dose-response curves to TRAIL, FasL, ETR1, ETR2 using primary ovarian cancer cell lines, save cell pellets.
b. Extract RNA and protein from cell pellets correlate with Six1 and DcR1 expression

Task 4. **Work performed:**

To date, analysis of 14 primary ovarian cancers have been performed. Results are listed in Table 1. Analysis of DcR1 has been halted as noted above and primary ovarian cancers and cell lines are currently being assayed for DcR2 expression. There is no clear correlation between Six1 status and TRAIL resistance yet although the sample size is small and analysis is on-going.

#	Age	stage	histology	Six1 fg/ng	Tumors?	TRAIL IC_{50}	ETR1 IC_{50}	ETR2 IC_{50}
159	43	IIIa	Clear Cell	0	No	R	R	R
140	48	IIIC	Serous	19	No	R	R	R
153	71	IV	Serous	20	No	R	R	R
137	58	IIIC	Serous	58	No	R	R	R
163	52	IIIC	Serous	135	No	2.5 ng/ml	R	200pg/ml
142	84	IV	Serous	137	No	R	R	R
160	60	IV	Mucinous	205	No	R	R	R
139	56	IIIC	Serous	209	No	R	R	R
150	65	IIIC	Serous	224	Yes	1	R	200
158	45	IIIC	Serous	295	Yes	5	R	600
162	57	IIIC	Serous	301	No	R	R	R
138	52	IIIC	Endo	310	Yes	R	R	R
164	52	IIIC	Serous	324	Yes	1	R	350
141	75	IIIC	Serous	906	No	R	R	R

KEY RESEARCH ACCOMPLISHMENTS:

- Generation of a Six1 over-expressing system in CaOv3 ovarian cancer cells
- Elimination of DcR1 as a downstream target of Six1 in this system
- Generation of an alternate hypothesis with DcR2 as a target of Six1

REPORTABLE OUTCOMES:

Qamar L, Thorburn A, Davidson SA, **Behbakht K**. Primary ovarian cancers are variably sensitive to TRAIL and Lecatumumab//the agonistic Antibody to TRAIL-Death Receptor 5 but not to Mapatumumab. (abstract) Presented at the 39th Annual Meeting of the Society of Gynecologic Oncologists, March 2008

CONCLUSIONS:

Too early. Studies are currently on-going.